Dkt No. PP00362.102
USSN: 09/674,183
PATENT

AMENDMENT

In the Specification:

Please amend the paragraph beginning at page 29, line 12 as follows:

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminium salts (alum), such as aluminum hydroxide, aluminium phosphate, aluminium sulphate, *etc*; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) MF59™ (WO 90/14837), containing 5% Squalene, 0.5% Tween™ 80, and 0.5% ~~Span~~ SPAN 85 (optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer (b) SAF, containing 10% Squalane, 0.4% ~~Tween~~ TWEEN 80, 5% pluronicblocked polymer L121, and thr-MDP either microfluidised into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), containing 2% Squalene, 0.2% ~~Tween~~ TWEEN 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Freund's complete and incomplete adjuvants (CFA & IFA); (5) cytokines, such as interleukins (*eg.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*eg.* IFNγ), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the efficacy of the composition. Alum and MF59™ are preferred.

Please amend the paragraph beginning at page 31, line 21 as follows:

~~Figure 1 is~~ Figures 1A and 1B show a schematic representation of the construction of the N6 protein.

Please amend the paragraph beginning at page 31, line 22 as follows:

~~Figure 2 illustrates~~ Figures 2A, 2B and 2C illustrate the N6 (Figure 2A) and N10 (Figures 2B and 2C) constructs and their respective DNA and amino acid sequences. The histidine tag, the flag peptide, the Fxa cutting site and the CD4+ T cell epitopes are underlined.

Please amend the paragraph beginning at page 32, line 20 as follows:

~~Figure 7 is~~ Figures 7A and 7B show a schematic representation of the N11 construct and its respective DNA and protein sequence. The hexahistidine tag, the flag peptide, the FXa cutting site, and the CD4+ T cell epitopes are underlined.

Please amend the paragraph beginning at page 32, line 23 as follows:

~~Figure 8 is~~ Figures 8A and 8B show a schematic representation of the N19 construct and its respective DNA and protein sequence. The hexahistidine tag, the flag peptide, the FXa cutting site, and the CD4+ T cell epitopes are underlined.

Please amend the paragraph beginning at page 33, line 4 as follows:

~~Figure 10~~ Figures 10A, 10B and 10C depict SDS-PAGE gels obtained from IMAC chromatography performed on N19 protein (Figure 10A) and N19 protein conjugated to Hib polysaccharide (Figure 10B), and western immunoblots of the N19 protein and the N19 protein conjugated to Hib polysaccharide using an anti-flag antibody (Figure 10C) as follows:

Please amend the paragraph beginning at page 33, line 5 as follows:

~~A~~ Figure 10A: SDS-Page and Coomassie staining. Analysis of the fractions obtained from IMAC chromatography performed to purify N19 protein. Lane a: prestained molecular weight markers. Lane b: flow through. Lanes from c to m: gradient fractions showing the purified N19 protein; the bands having a molecular weight lower than N19 and visible in the overloaded lanes f, g, and h represents degradation products

of the N19 protein.

Please amend the paragraph beginning at page 33, line 10 as follows:

~~B~~ Figure 10B: SDS-Page and Coomassie staining. Analysis of the fractions obtained from IMAC chromatography of the N19 conjugated to Hib polysaccharide. All N19 protein resulted to be conjugated, as judged by the high molecular weight of the conjugate and by the absence of 43.000 kDa unconjugated N19 protein.

Please amend the paragraph beginning at page 33, line 14 as follows:

~~C~~ Figure 10C: The same conjugate samples used in picture B were subjected to western immunoblot using an anti-flag antibody. Also here it can be appreciated that all N19 protein migrated as a very high molecular weight after conjugation to Hib polysaccharide, and that there is not unconjugated N19 protein migrating at 43.000 kDa.

Please amend the paragraph beginning at page 37, line 1 as follows:

After nucleotide sequencing of the selected clones, a clone named pEMBLN6 was shown to contain six different T cell epitopes with no repetitive sequences. The N6 insert was then PCR-amplified and transferred to pTrc-His expression vector (Invitrogen) using standard techniques (Sambrook *et al.*, 1989). The generation of the N6 expressing plasmids is summarised in ~~Figure 1~~ Figures 1A and 1B.

Please amend the paragraph beginning at page 38, line 24 as follows:

Then, the resin was packed in a column and washed with buffer A. Guanidinium-HCl was removed from the column by washing with buffer B (8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris base) pH 8. After a wash with buffer B pH 6.5, recombinant proteins were eluted with a 20 ml buffer B gradient from ph 6.5 to pH 4. The ~~factions~~ fractions containing the purified recombinant proteins were pooled and dialysed against PBS, pH 7.2. Proteins were analysed by SDS-PAGE and protein content was determined using the Bradford method. Alternatively, cell pellets obtained from *E coli* cultures wer

solubilized by heating at 37 °C in buffer A, the lysates were centrifuged to 15.000 g for 20 min. The supernatants were subjected to column chromatography on ~~Nickel activated Chelating Sepharose Fast Flow~~ NICKEL ACTIVATED CHELATING SEPHAROSE FAST FLOW (Pharmacia). After a wash with buffer A and a wash with buffer B, pH 7, the proteins were separated by collecting fractions from a 0-200 mM gradient of Imidazole in buffer B, pH 7. The fractions containing the purified recombinant proteins (as judged by SDS-PAGE and Coomassie staining) were pooled and dialysed against PBS, pH 7.2.

Please amend the paragraph beginning at page 39, line 21 as follows:

The permeate obtained from 10 kDa UF was loaded, at a linear flow rate of 150 cm/h, onto a ~~Q-Sepharose Fast Flow~~ Q-SEPHAROSE FAST FLOW column [10 cm (ID); 5,5 cm (h)] equilibrated with 0.08 M NaCl/0.05 M sodium acetate pH 6. After adsorption, low molecular weight fragments (up to 5 repeats) were removed by washing the column with 10 column volumes of equilibrating buffer and then eluted with 3 column volumes of 0.5 M NaCl/0.005M sodium acetate pH 6. The eluate was 0.2 µm filtered and then analysed for avDp and ion exchange analytical chromatography. AvDP resulted at 17.3, ion exchange analytical chromatography on ~~Mono~~ MONO Q HR 5/5 revealed the absence of any small fragments until DP 5.

Please amend the paragraph beginning at page 40, line 1 as follows:

To introduce a terminal amino group, reductive amination was then performed; to the fractionated Hib oligosaccharide obtained from ~~Q-Sepharose~~ Q-SEPHAROSE chromatography, ammonium chloride 35mg/ml and sodium cyanoboroidride 12 mg/ml final concentrations were added. After stirring, the solution was 0.2 µm filtered and incubated at 37 °C for 120 hours. The amino oligosaccharide was then purified from excess of reagents by precipitation with 95° EtOH (81° final concentration) in the cold for 15-20 hours. The precipitated oligosaccharide was then recovered by centrifugation,

solubilized in NaCl 0.4M using approximately 1/4 of the starting volume and precipitated again at 81° EtOH in the cold for 15-20 hours.

Please amend the paragraph beginning at page 41, line 1 as follows:

33.4nmoles of recombinant carrier protein and 669nmoles of activated Hib oligosaccharide in a final volume of 0.5 ml 10mM phosphate buffer, pH 7, were gently stirred overnight at RT and brought up to 5ml 1M $(NH_4)2SO_4$, 10mM phosphate pH7. The sample was subjected to FPLC on a 1ml ~~Phenyl Sepharose~~ PHENYL SEPHAROSE 5/5 HR column (Pharmacia). 1ml fractions were collected both during washing (1M $(NH_4)2SO_4$, 10mM phosphate, pH 7) and elution (10mM phosphate, pH 7). Two peaks corresponding to the non-adsorbed material and to the eluted material were obtained. The pooled fractions corresponding to the non-adsorbed material and the pooled fractions corresponding to the elution peak were subjected to protein and ribose content determination and to SDS-PAGE and Western blot analysis.

Please amend the paragraph beginning at page 43, line 21 as follows:

~~Nunc-Maxisorp~~ NUNC MAXISORP 96-well flat-bottomed plates were coated by overnight incubation at 4°C with 1µg/ml (as polysaccharide) of a human serum albumin (HSA) and *H. influenzae* type b polysaccharide conjugate (HSA-Hib). After washing, wells were over-coated using 1% (w/v) gelatin in PBS, pH 7.2 for 3 additional hours at 37°C. Serum samples were diluted 1:50 in 5mM phosphate buffer, pH 7.2 containing 75mM NaCL 1% (w/v) BSA and 0.05% (w/v) ~~Tween-20~~ TWEEN-20 and dispensed in duplicate into the wells. Sera from untreated mice were pooled and diluted 1:50 as above and dispensed into 8 wells. After overnight incubation at 4°C, plates were washed three times with 5mM phosphate buffer, pH 7.2 containing 75mM NaCl and 0.05% (w/v) ~~Tween 20~~ TWEEN-20. Then, alkaline phosphate-conjugated goat IgG anti-mouse lgG diluted 1:1000 and 5mM phosphate buffer, pH 7.2 containing 75mM NaCl. 1% (w/v) BSA and 0.05% (w/v) ~~Tween 20~~ TWEEN-20 were added to each well, and incubated 3 hours at 37°C.

Please amend the paragraph beginning at page 45, line 3 as follows:

The clone expressing N6 protein comprised the plasmid pTrc-N6 transformed in the Top10 *E. coli* strain. As deduced from plasmid DNA sequencing, this plasmid ~~code~~ coded for a protein having an hexahistidine amino terminal tail followed in sequence by a flag peptide, a FXa site, and the following T cell epitopes: P23TT, P32TT, P21TT, PfT3, P30TT, and P2TT. All the epitopes were spaced by a KG ~~aminoacid~~ amino acid sequence (~~Fig.2~~ Figure 2A).

Please amend the paragraph beginning at page 45, line 8 as follows:

The two clones that produced N10 protein were the Top10 *E. coli* strain containing the plasmid pTrc-N10, and the TGI *E. coli* strain containing the plasmid pQE-N10. Both these clones contained the N6 coding sequence fused to a carboxy terminal sequence coding for four additional T cell epitopes which were in the order: HBVnc, HA, HBsAg, and MT (~~Fig 2~~ Figures 2B-2C).

Please amend the paragraph beginning at page 45, line 13 as follows:

The clone that produced N11 protein comprised the plasmid pTrc-N10 transformed in the Top10 *E. coli* strain. As deduced from plasmid DNA sequencing, this plasmid ~~code~~ coded for a protein consisting ~~in~~ of the N10 sequence fused to a carboxy terminal sequence coding for the HSP70 T cell epitope (~~Fig. 7~~ Figures 7A-7B).

Please amend the paragraph beginning at page 46, line 29 as follows:

As it was for the N10 protein, also the expression of N19 protein was improved by changing the expression vector (from pTrc-His to pQE30) and the host strain (from Top10 to TG1). TGI(QE-N19) was used to purify N19 polyepitope protein. By subjecting solubilised inclusion bodies to IMAC chromatography, we purified (see figure 10A) 5.42 mg of N19 protein from one litre of flask culture. The identity of N19 was identified in SDS-Page as an induced band having the expected molecular weight, in immuno western

blot using an anti-flag antibody, and was also deduced after plasmid DNA sequencing (~~figure 8~~ Figures 8A-8B).